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# Inhibition and Enhancement of Oncogenic Cell Transformation in C3H10T1/2CL8 Cells

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by

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16. ABSTRACT

During the last several years, we have been studying the effects of chemicals on the inhibition and enhancement of oncogenic cell transformation in C3H10T1/2C18 mouse embryo fibroblasts (C3H10T1/2 cells). Our findings, summarized in Table 1, indicate that many diverse chemicals can affect oncogenic cell transformation. In general, their mechanisms of action seem to be at the level of the enzymes which are involved in the metabolic activation of the carcinogens used to transform the cells. Specifically, 7,8-benzoflavone, 1,2-naphthoquinone, and phenanthrene-9,10- quinone inhibit cell transformation by inhibiting cytochrome P-450 mediated oxidation (11, 13). Benz(a)anthracene, 5,6-benzoflavone, phenobarbital, and pregnenolone-16-α-carbonitrile enhance cell transformation by inducing the complex of cytochrome P-450 mixed-function oxidases and increase the ability of C3H10T1/2 cells to metabolize carcinogens (14). Cyclohexane, cyclohexane oxide, 1,2-dihydronaphthalene, stryene oxide and 1,2,3,4-tetrahydronaphthalene-1,2-oxide enhance cell transformation by inhibiting epoxide hydratase and thus allow increased levels of active arene oxides to accumulate in the cells (13).

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# INHIBITION AND ENHANCEMENT OF ONCOGENIC CELL TRANSFORMATION IN C3H10T1/2CL8 CELLS

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During the last several years, we have been studying the effects of chemicals on the inhibition and enhancement of oncogenic cell transformation in C3H10T1/2Cl8 mouse embryo fibroblasts (C3H10T1/2 cells). Our findings, summarized in Table 1, indicate that many diverse chemicals can affect oncogenic cell transformation. In general, their mechanisms of action seem to be at the level of the enzymes which are involved in the metabolic activation of the carcinogens used to transform the cells. Specifically, 7,8-benzoflavone, 1,2-naphthoquinone, and phenanthrene-9, 10-quinone inhibit cell transformation by inhibiting cytochrome P-450 mediated oxidation (11,13). Benz(a)anthracene, 5,6-benzoflavone, phenobarbital, and pregnenolone-16-α-carbonitrile enhance cell transformation by inducing the complex of cytochrome P-450 mixed-function oxidases and increase the ability of C3H10T1/2 cells to metabolize carcinogens (14). Cyclohexene, cyclohexene oxide, 1,2-dihydronaphthalene, styrene oxide and 1,2,3,4-tetrahydronaphthalene-1, 2-oxide enhance cell transformation by inhibiting epoxide hydratase and thus allow increased levels of active arene oxides to accumulate in the cells (13).

The mixed-function oxidases in C3H10T1/2 cells are efficient at metabolizing polycyclic aromatic hydrocarbons (PAH) such as 3-methylcholanthrene [3MC] (13), benzo(a)pyrene [B(a)P] (14), and cyclopenta(cd)pyrene (6) and are not efficient at metabolizing 2-acetylaminofluorene (15). These results are in concert with the observation that PAH can transform C3H10T1/2 cells while 2-acetylaminofluorene cannot (6, 9, 12-16).

*TABLE 1. Inhibitors and enhancers of oncogenic transformation in C3H10T1/2 cells*[a]

| Chemical | Carcinogen | Effect | Reference |
|---|---|---|---|
| Benz(a)anthracene | B(a)P | enhancement | 14 |
| | 3MC | enhancement | 13 |
| 5,6-Benzoflavone | B(a)P | enhancement | 14 |
| 7,8-Benzoflavone | 3MC | inhibition | 13 |
| | B(a)P | inhibition | Ub |
| Cyclohexene | 3MC | enhancement | 13 |
| Cyclohexene oxide | 3MC | enhancement | 13 |
| 1,2-Dihydronaphthalene | 3MC | enhancement | 13 |
| 1,2-Naphthoquinone | B(a)P | inhibition | 11 |
| Phenanthrene-9, 10-quinone | B(a)P | inhibition | 11 |
| Phenobarbital | B(a)P | enhancement | 14 |
| Pregnenolone-16-α carbonitrile | B(a)P | enhancement | 14 |
| Styrene oxide | 3MC | enhancement | 13 |
| 1,2,3,4-Tetrahydro-naphthalene-1,2-oxide | 3MC | enhancement | 13 |

[a]Data taken from the author's publications.
[b]Unpublished data.

During our studies of cell transformation and metabolism of indirect-acting carcinogens, we observed, as had others, that C3H10T1/2 cells could not be transformed by direct-acting alkylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or the k-region oxides of most PAH. The only direct-acting carcinogens reported to transform C3H10T1/2 cells were

cyclopenta(cd)pyrene-3, 4-oxide (6) and N-hydroxy-2-acetylaminofluorene (15), however these agents were weak in activity. In some of our experiments with enzyme inducers we noticed that when dishes containing cells were treated with carcinogens several days after seeding they produced more foci than when they were treated one day after seeding as described in the original procedure of Reznikoff et al. (16). We investigated this observation by treating separate groups of dishes of C3H10T1/2 cells with B(a)P on various days after seeding (Fig. 1). We found that the production of foci increased with increasing time interval between seeding and treatment up to 5 days after seeding. After 5 days foci production decreased. When this type of experiment was repeated with MNNG, similar results were obtained, except the decrease was not observed up to 8 days after seeding (data not shown). This was the first time

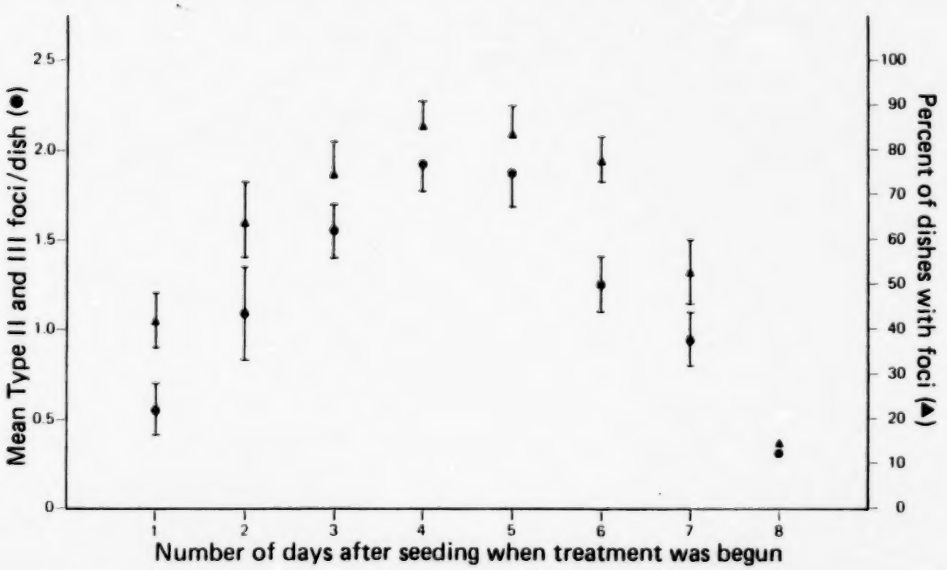


FIG. 1. Temporal relationship between day of treatment and oncogenic transformation in C3H10T1/2 cells. Cells were seeded on day 10 and treated with 1 μg/ml B(a)P for 24 hr on subsequent days.

MNNG had been reported to transform C3H10T1/2 cells (12). Using the modified protocol of treating 5-day old cultures of C3H10T1/2 cells we also obtained cell transformation with seven other carcinogens (Table 2). Several carcinogens have been examined and have been found to be refractory to transforming C3H10T1/2 cells using this procedure (Table 2), probably due to the inability of C3H10T1/2 cells to metabolically activate them.

*TABLE 2. Carcinogens evaluated in the 5-day treatment protocol using C3H10T1/2 cells.*[a]

| Chemical | Result |
|---|---|
| 2-Acetylaminofluorene | no transformation |
| Aflatoxin $B_1$ | transformation |
| B(a)P | transformation |
| N,N'-Dimethylaminoazobenzene | no transformation |
| 7,12-Dimethylbenz(a)anthracene | transformation |
| Epichlorohydrin | transformation |
| Ethyl nitrosourea | transformation |
| 3MC | transformation |
| MNNG | transformation |
| Nickel sulfate | no transformation |
| 4-Nitroquinoline-N-oxide | transformation |
| Propane sultone | transformation |
| Urethane | no transformation |
| Vinyl carbamate | no transformation |

[a]Data from the author's laboratory.

Recently we have been examining the phenomena of inhibition of cell transformation in C3H10T1/2 cells by chemicals which do not exert their action by affecting the microsomal mixed-function

oxidases. This paper will document our findings and describe studies designed to ascertain the operable mechanisms of action.

## MATERIALS AND METHODS

C3H10T1/2 cells derived by Reznikoff et al. (17) were cultured according to previously published procedures (12,16). In general, cell transformation studies were performed by seeding 1000 cells/dish in Eagle's basal medium with Earle's salts and L-glutamine and supplemented with 10% heat-inactivated fetal calf serum. After the chemical treatment, the cells were washed and the medium replaced with fresh medium containing Garramycin sulfate, 25 µg/ml. At confluence, the fetal calf serum concentration was reduced to 5% (2). Medium changes were performed weekly. At the end of the experiments the cells were washed with 0.85% NaCl, fixed with methanol and stained with Giemsa. Both type II and type III foci were scored according to previously described criteria (16). Cytotoxicity determinations were performed using 200 cells/dish and the dishes were fixed and stained 10-12 days after treatment.

## RESULTS AND DISCUSSION

We are interested in enhancing the chemical transformation of C3H10T1/2 cells by various techniques. We reasoned that cell transformation experiments, especially those performed with C3H10T1/2 cells, employ single exposures, unlike the chronic exposure tumorigenesis experiments. We therefore treated C3H10T1/2 cells with carcinogens at several times throughout the transformation experiment. Our initial findings were that cells treated on day 1 and day 8 with B(a)P (each treatment at 0.5 µg/ml) produced more total type II and III foci than cells treated with 1 µg/ml B(a)P on day 1 (Table 3). However, C3H10T1/2 cells treated with B(a)P at 0.5 µg/ml on days 1,8 and 15 produced less total foci and fewer dishes with foci than dishes treated with B(a)P at 1.5 µg/ml on day 1. The cells treated on days 1,8 and 15 also produced fewer foci and dishes with foci than B(a)P treated cells on days 1 and 8. These results suggested that C3H10T1/2 cells in log phase growth produced more transformation when treated with split concentrations of B(a)P. They also suggested that B(a)P initiated cells, when at confluence, were inhibited in expressing transformation when treated again with B(a)P.

The time course of inhibition was explored further by treating B(a)P initiated cells at various times after reaching confluence. Each B(a)P treatment consisted of a 24 hr exposure

*TABLE 3. Response of B(a)P initiated C3H10T1/2 cells to subsequent B(a)P treatment*

| | Treatment Day(s) | | | | | |
|---|---|---|---|---|---|---|
| | 1 | | 1 and 8 | | 1,8 and 15 | |
| B(a)P, μg/ml | No. Foci[a] | % Dishes with Foci[a] | No. Foci[a] | % Dishes with Foci[a] | No. Foci[a] | % Dishes with Foci[a] |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.5 | 34 | 58 | 75 | 81 | 26 | 50 |
| 1.0 | 52 | 78 | 96 | 83 | | |
| 1.5 | 56 | 83 | | | | |

[a]Total of type II and type III foci per 24 dishes scored per treatment group. Treatment time was 24 hr for each treatment.

to 1 μg/ml. C3H10T1/2 cells treated a second time with B(a)P from day 14 to day 26 did not produce any transformed foci (Table 4). A small number of foci were observed in dishes treated on days 1 and 33.

In order to determine whether this inhibition was a temporary event or a result of delayed cytotoxicity, cells treated on day 1 or days 1 and 22 were held for longer times before scoring (Table 5). Cells treated on days 1 and 22 normally scored at week 6 again produced no foci. Cells treated on day 1, and held 1 to 3 weeks beyond week 6 (weeks 7 to 9) produced increasing numbers of foci. However cells treated on days 1 and 22 and held to week 9 produced only 2 foci/ 24 dishes. The extent of inhibition did not change with the week of scoring indicating that the change observed was not temporary.

Several experiments were performed in order to probe the mechanisms of this effect. Selective cytotoxic effects of B(a)P on transformed cells were determined by employing a reconstruction procedure. Transformed C3H10T1/2 cells, 58-3MC cells, were co-seeded with C3H10T1/2 cells at ratios of 8 58-3MC

*TABLE 4. Inhibition of B(a)P- induced transformation as a function of time of second treatment*[a]

| Time of Second Treatment[b] (day) | Total Foci[c] | % Dishes with Foci[c] |
|---|---|---|
| 14 | 0 | 0 |
| 16 | 1 | 4 |
| 22 | 0 | 0 |
| 26 | 0 | 0 |
| 33 | 4 | 3 |

[a]C3H10T1/2 cells were treated on day 1 with B(a)P (1 μg/ml) for 24 hr.
[b]Second treatment was with B(a)P (1 μg/ml) for 24 hr.
[c]Total type II and III foci per 24 dishes scored.

cells to 100,000 C3H10T1/2 cells and 16 58-3MC cells to 100,000 C3H10T1/2 cells. After attaining confluence, the dishes were treated with B(a)P or MNNG, 1 μg/ml, for 24 hr. The dishes were fixed and stained 7 days later. Of 8 or 16 transformed cells seeded all were recovered (Table 6). B(a)P killed approximately 70% of the 58-3MC cells seeded while MNNG killed approximately 50%. A second study was performed to investigate the cytotoxic effects of the second B(a)P treatment. Cells treated on days 1 and 22 were reseeded on day 23 and the plating efficiency measured (Table 7). A 92% decrease in plating efficiency was observed when compared to cells treated on day 1 and reseeded on day 23. These results suggest that many initiated cells treated on day 22 are not likely to survive the treatment. However, since it only takes one initiated cell to form a transformed colony and since B(a)P does not kill all the transformed cells, cytotoxicity cannot explain the observations of complete inhibition of transformation.

Many chemicals have been reported to inhibit cell transformation: actinomycin D (5); allylisopropylacetamide (9); antibiotics (4); antifungals (16); ascorbic acid (1); dexamethasone (8); isopropylvaleramide (9); phosphodiesterase

*TABLE 5. Inhibition of B(a)P-induced transformation by B(a)P as a function of time of scoring*[a]

| Week of Scoring | No. Foci/24 Dishes Treatment Days: Day 1 | Days 1, 22 | %Inhibition of Transformation |
|---|---|---|---|
| 6 | 34 | 0 | 100 |
| 7 | 68 | 2 | 97 |
| 8 | 84 | 2 | 98 |
| 9 | 82 | 2[b] | 98 |

[a]B(a)P concentration was 1 μg/ml for each treatment. Treatment time was 24 hr for each treatment.

[b]Four foci appearing in the Day 22 controls have been subtracted from this total.

inhibitors (3); protease inhibitors (7); retinoids (10) and; serum components (2). Although the mechanisms of action of these chemicals are not known, a number of these chemicals have been examined for their ability to inhibit the mixed-function oxidases and have been found to be inactive in this regard. It has been proposed that some of these chemicals may exert their action by affecting receptors involved in the transformation process (10). We are currently evaluating this mechanism with regard to our observations on the inhibition of cell transformation of C3H10T1/2 cells by B(a)P.

*TABLE 6. Determination of the cytotoxic effects of the second B(a)P treatment on transformed cells using a reconstruction procedure*[a]

| No. 58-3MC Cells Seeded: | 8 | 16 | 8 | 16 |
|---|---|---|---|---|
| Treatment | Foci/Dish[b] | | % Survival | |
| Acetone | 8.6 | 14.8 | 100 | 100 |
| B(a)P (1 μg/ml) | | | 33.5 | 31.8 |
| MNNG (1 μg/ml) | | | 47.8 | 51.5 |

[a]100,000 C3H10T1/2 cells were seeded with 8 or 16 58-3MC cells per 60 mm Petri dishes. After attaining confluence, the cultures were treated with B(a)P or MNNG for 24 hr. The cultures were fixed and stained 7 days later and scored for 58-3MC cell foci.

[b]Mean of 104 individual determinations. The coefficient of variation was <15%.

*TABLE 7. Plating efficiency of initiated C3H10T1/2 cells treated on day 22 with B(a)P*[a]

| Treatment Group | Plating Efficiency,% Mean +/- SD[b] |
|---|---|
| Acetone (Days 1,22) | 13.3 +/- 2.5 |
| B(a)P (Day 1) | 15.1 +/- 2.1 |
| B(a)P (Days 1,22) | 1.2 +/- 0.7 |

[a]Cells were treated with B(a)P (1 μg/ml) for 24 hr on days 1 or 1 and 22 (except for the acetone controls). On day 23, the cells were trypsinized and reseeded at 200 cells/dish. Colony counts were performed 7 days later.

[b]The number of replicates for each determination was 24.

## DEDICATION

This paper is dedicated to the memory of Charles Heidelberger, teacher, mentor and friend.

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